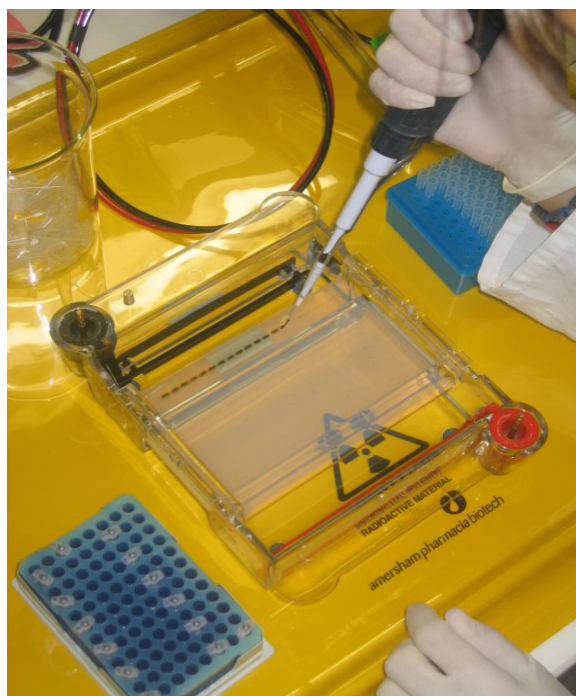


Report on the Verification of the Performance of MON89034,1507, MON88017 and 59122 Maize Event-specific Methods on the Maize Event MON89034 x 1507 x MON88017 x 59122 Using Real-time PCR

Validation Report and Protocols

E. Luque-Perez, A. Bevilacqua, S. Larcher, G. Pinski, M. Mazzara, G. Van den Eede



EUR 24706 EN - 2010

The mission of the JRC-IHCP is to protect the interests and health of the consumer in the framework of EU legislation on chemicals, food, and consumer products by providing scientific and technical support including risk-benefit assessment and analysis of traceability.

European Commission
Joint Research Centre
Institute for Health and Consumer Protection

Contact information

Address: Molecular Biology and Genomics Unit
E-mail: JRC-BGMO@ec.europa.eu
Tel.: +39 0332 789379
Fax: +39 0332 786159

<http://ihcp.jrc.ec.europa.eu/>
<http://www.jrc.ec.europa.eu/>

Legal Notice

Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use which might be made of this publication.

***Europe Direct is a service to help you find answers
to your questions about the European Union***

**Freephone number (*):
00 800 6 7 8 9 10 11**

(*) Certain mobile telephone operators do not allow access to 00 800 numbers or these calls may be billed.

A great deal of additional information on the European Union is available on the Internet.
It can be accessed through the Europa server <http://europa.eu/>

JRC62814
EUR 24706 EN
ISBN 978-92-79-19082-7
ISSN 1018-5593
doi:10.2788/31795

Luxembourg: Publications Office of the European Union

© European Union, 2010

Reproduction is authorised provided the source is acknowledged

Printed in Italy



Report on the Verification of the Performance of MON 89034, 1507, MON 88017 and 59122 Event-specific Methods on the Maize Event MON 89034 x 1507 x MON 88017 x 59122 Using Real-Time PCR

1 July 2010

**Joint Research Centre
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit**

Executive Summary

The European Union Reference Laboratory for GM Food and Feed (EURL-GMFF), established by Regulation (EC) No 1829/2003, has carried out a verification study to assess the performance of four quantitative event-specific methods on the maize event MON 89034 x 1507 x MON 88017 x 59122 (unique identifier MON-89034-3 x DAS-01507-1 x MON-88017-3 x DAS-59122-7) which combines the MON 89034, 1507, MON 88017 and 59122 transformation events. The four methods have been validated individually on single-trait events, to detect and quantify each event in maize samples. This study was conducted according to internationally accepted guidelines ^(1, 2).

In accordance to Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and to Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Monsanto and Dow AgroSciences provided the detection methods and the control samples: genomic DNA from homogenised seeds of MON 89034 x 1507 x MON 88017 x 59122 maize (258-4CC, 258-4JJ, 258-4B, 258-4N) and from homogenised seeds of conventional maize (10001262-V). The EURL-GMFF prepared the verification samples (calibration samples and blind samples at different GM percentages).

The results of the verification study were evaluated with reference to ENGL method performance requirements (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>) and to the validation results on the individual parental events (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>).

The results of this EURL-GMFF verification study are publicly available at <http://gmo-crl.jrc.ec.europa.eu/>.

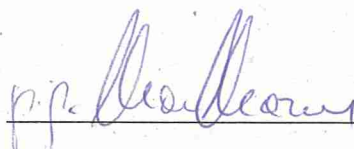
Drafted by:

E. Luque-Perez (scientific officer)



Report Verification:

1) L. Bonfini (scientific officer)

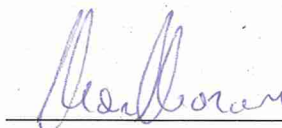


2) M. Ermolli (scientific officer)



Scientific and technical approval:

M. Mazzara (scientific officer)



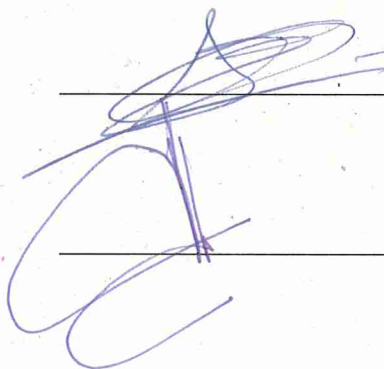
Compliance with EURL Quality System:

S. Cordeil (quality manager)



Authorisation to publish:

G. Van den Eede (head of MBG unit)



Address of contact laboratory:

European Commission, Joint Research Centre (JRC)
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit
European Union Reference Laboratory for GM Food and Feed
Via E. Fermi 2749, I-21020 Ispra (VA) - Italy

Report on Steps 1-3 of the Validation Process

Monsanto and Dow AgroSciences submitted the detection methods and control samples of the maize event MON 89034 x 1507 x MON 88017 x 59122 (unique identifier MON-89034-3 x DAS-Ø15Ø7-1 x MON-88Ø17-3 x DAS-59122-7) under Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The European Union Reference Laboratory for GM Food and Feed (EURL-GMFF), following reception of the documentation and material, including control samples, (step 1 of the validation process) carried out the scientific assessment of documentation and data (step 2) in accordance with Commission Regulation (EC) No 641/2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation" and according to its operational procedures ("Description of the EURL-GMFF Validation Process", <http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>).

The scientific assessment focused on the method performance characteristics assessed against the method acceptance criteria set out by the European Network of GMO Laboratories and listed in the "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (<http://gmo-crl.jrc.ec.europa.eu/doc/Method%20requirements.pdf>) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, two scientific assessments were performed and one request of complementary information was addressed to the applicant. Upon reception of the complementary information, the scientific assessment of the detection method for maize MON 89034 x 1507 x MON 88017 x 59122 was positively concluded in January 2009.

The event-specific detection methods for the four maize lines hosting the single events MON 89034, 1507, MON 88017 and 59122 were validated by the EURL-GMFF following the conclusion of the respective international collaborative studies and the publication of the validation reports (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>). Hence, the detection methods applied on the maize event MON 89034 x 1507 x MON 88017 x 59122 did not undergo a full validation process. The EURL-GMFF performed a verification of the detection methods to verify that they exhibit a comparable performance on samples of event MON 89034 x 1507 x MON 88017 x 59122 combining the four traits (as provided in accordance to Annex I.2.C.2 of Commission Regulation (EC) No 641/2004).

In February 2009, the EURL-GMFF concluded the experimental verification of the methods (step 3, experimental testing of the samples and methods) by quantifying, with each specific method, five blind GM-levels within the range 0.09%-8% on a copy number basis. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, trueness and repeatability of the quantification were within the limits established by the ENGL.

A Technical Report summarising the results of tests carried out by the EURL-GMFF (step 3) is available on request.

Content

1. INTRODUCTION	5
2. MATERIALS.....	6
3. EXPERIMENTAL DESIGN	6
4. METHODS	7
5. DEVIATIONS REPORTED	7
6. RESULTS	7
PCR EFFICIENCY AND LINEARITY	7
7. METHOD PERFORMANCE REQUIREMENTS.....	10
8. COMPARISON OF METHOD PERFORMANCE BETWEEN EVENT MON 89034 X 1507 X MON 88017 X 59122 AND THE SINGLE TRAIT EVENTS	11
9. CONCLUSIONS.....	14
10. QUALITY ASSURANCE	14
11. REFERENCES	14
12. ANNEX 1: METHOD ACCEPTANCE CRITERIA AND METHOD PERFORMANCE REQUIREMENTS AS SET BY THE EUROPEAN NETWORK OF GMO LABORATORIES (ENGL)	15

1. Introduction

Monsanto and Dow AgroSciences submitted the detection methods and control samples of the maize event MON 89034 x 1507 x MON 88017 x 59122 (unique identifier MON-89034-3 x DAS-Ø15Ø7-1 x MON-88Ø17-3 x DAS-59122-7) under Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The European Union Reference Laboratory for GM Food and Feed, established by Regulation (EC) 1829/2003, carried out a verification of the four event-specific methods for the detection and quantification of MON 89034, 1507, MON 88017 and 59122 in the MON 89034 x 1507 x MON 88017 x 59122 maize event combining the four traits. The single methods had been previously validated by international collaborative studies on the single-trait maize events (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>).

Upon reception of methods, samples and data (step 1), the EURL-GMFF carried out the assessment of the documentation (step 2) and the verification of the methods (step 3) according to the requirements of Regulation (EC) 641/2004 and following EURL-GMFF procedures. The EURL-GMFF method verification was concluded in February 2009.

A method submitted by the applicant for DNA extraction from maize seeds was also evaluated by the EURL-GMFF in order to confirm its performance characteristics. The protocol for DNA extraction is available at <http://gmo-crl.jrc.ec.europa.eu/>.

The procedure of verification consisted of a quantitative real-time Polymerase Chain Reaction (PCR). The methodology consists of four event-specific real-time quantitative TaqMan® PCR procedures for the determination of the relative content of events MON 89034, 1507, MON 88017 and 59122 DNA to total maize DNA in the MON 89034 x 1507 x MON 88017 x 59122 maize event. The procedures are simplex systems, in which the events MON 89034, 1507, MON 88017 and 59122 were quantified in reference to the maize *hmg* (*high mobility group*) taxon-specific endogenous gene.

The study was carried out in accordance to the following internationally accepted guidelines:

- ISO 5725: 1994
- The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies".

2. Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from homogenised seeds of MON 89034 x 1507 x MON 88017 x 59122 maize (258-4CC, 258-4JJ, 258-4B, 258-4N),
- genomic DNA extracted from homogenised seeds of conventional maize (10001262-V).

in accordance to the provisions of Regulation (EC) No 1829/2003, Art 2.11 [control sample defined as "the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample)"].

Samples containing mixtures of MON 89034 x 1507 x MON 88017 x 59122 and non-GM maize genomic DNA at different GMO levels were prepared in a constant amount of total maize DNA.

The validated methods for the individual MON 89034, 1507, MON88017 and 59122 events were applied in the verification as published and available at <http://qmo-crl.jrc.ec.europa.eu/statusofdoss.htm>.

In Table 1 are reported the five GM levels used in the verification of the MON 89034, 1507, MON 88017 and 59122 methods.

Table 1. MON 89034, 1507, MON 88017 and 59122 GM contents (%) in maize event MON 89034 x 1507 x MON 88017 x 59122

MON 89034 (GM DNA / Non-GM DNA x 100)	1507 (GM DNA / Non-GM DNA x 100)	MON 88017 (GM DNA / Non-GM DNA x 100)	59122 (GM DNA / Non-GM DNA x 100)
0.09	0.1	0.09	0.1
0.4	0.5	0.5	0.4
0.9	0.9	0.9	0.9
3.0	2.0	5.0	2.0
8.0	5.0	8.0	4.5

3. Experimental design

Eight runs for each event-specific method were carried out. In each run, samples were analysed in parallel with both the GM-specific system and the target taxon-specific assay (*hmg*). Five GM levels per run were examined and two replicates for each GM level were analysed. PCR analysis was performed in triplicate for all samples. In total, for each method (MON 89034, 1507, MON 88017 and 59122), the quantification of the five GM levels was performed as an average of sixteen replicates per GM level.

4. Methods

To detect MON 89034, 1507, MON 88017 and 59122 in maize event MON 89034 x 1507 x MON 88017 x 59122, four specific fragments of the integration regions of 77 bp, 58 bp, 95 bp and 86 bp respectively, were amplified using specific primers.

For relative quantification of events MON 89034, 1507, MON 88017 and 59122, a maize-specific target-taxon system which amplifies a 79 bp fragment of the maize gene *hmg* (high mobility group), using *hmg* gene-specific primers and a *hmg* gene-specific probe labelled with FAM and TAMRA, were used.

Standard curves are generated for each GM specific system (MON 89034, 1507, MON 88017 and 59122), by plotting the Ct values of the calibration samples against the logarithm of the DNA copy numbers of MON 89034, 1507, MON 88017 or 59122, and fitting a linear regression into these data. Thereafter, the normalised Ct values of the blind samples are measured and, by means of the regression function, the relative amount of MON 89034, 1507, MON 88017 or 59122 DNA is estimated.

Detailed information on standard curve samples preparation is available at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>.

5. Deviations reported

For the 1507 method, only seven runs were carried out, instead of eight. Therefore, the quantification of the five GM levels was performed on fourteen replicates per GM level instead of sixteen.

6. Results

PCR efficiency and linearity

PCR efficiency was calculated using the formula $[10^{(-1/\text{slope})} - 1] \times 100$, and the R^2 (expressing the linearity of the regression) is reported for all PCR systems in the eight runs. Values of the standard curves slopes for MON 89034, 1507, MON 88017 and 59122 methods are presented in Tables 2, 3, 4 and 5, respectively.

Table 2. Values of standard curve slope, PCR efficiency and R^2 of the MON 89034 method on event MON 89034 x 1507 x MON 88017 x 59122.

Run	MON 89034			<i>hmg</i>		
	Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	-3.48	94	1.00	-3.21	105	0.99
2	-3.55	91	1.00	-3.20	105	1.00
3	-3.57	91	1.00	-3.13	109	0.99
4	-3.47	94	1.00	-3.19	106	1.00
5	-3.56	91	1.00	-3.23	104	0.99
6	-3.43	96	1.00	-3.17	107	1.00
7	-3.56	91	1.00	-3.14	108	1.00
8	-3.39	97	1.00	-3.20	105	1.00
Mean	-3.50	93	1.00	-3.18	106	1.00

Table 3. Values of standard curve slope, PCR efficiency and R^2 of the 1507 method on event MON 89034 x 1507 x MON 88017 x 59122.

Run	1507			<i>hmg</i>		
	Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	-3.21	105	0.99	-3.15	108	1.00
2	-3.24	103	0.99	-3.10	110	1.00
3	-3.15	108	0.99	-3.14	108	1.00
4	-3.13	109	1.00	-3.11	110	1.00
5	-3.20	105	0.99	-3.15	108	1.00
6	-3.35	99	0.99	-3.10	110	1.00
7	-3.21	105	0.99	-3.15	108	1.00
Mean	-3.22	105	0.99	-3.13	109	1.00

Table 4. Values of standard curve slope, PCR efficiency and R^2 of the MON 88017 method on event MON 89034 x 1507 x MON 88017 x 59122.

Run	MON 88017			<i>hmg</i>		
	Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	-3.16	107	1.00	-3.16	107	1.00
2	-3.36	99	1.00	-3.20	105	1.00
3	-3.27	102	1.00	-3.18	106	1.00
4	-3.25	103	1.00	-3.20	105	1.00
5	-3.23	104	1.00	-3.16	107	0.99
6	-3.31	100	1.00	-3.16	107	1.00
7	-3.29	101	1.00	-3.21	105	0.99
8	-3.39	97	0.99	-3.19	106	1.00
Mean	-3.28	102	1.00	-3.18	106	1.00

Table 5. Values of standard curve slope, PCR efficiency and R^2 of the 59122 method on event MON 89034 x 1507 x MON 88017 x 59122.

Run	59122			<i>hmg</i>		
	Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	-3.48	94	1.00	-3.25	103	1.00
2	-3.43	96	0.99	-3.31	100	1.00
3	-3.22	104	1.00	-3.28	102	1.00
4	-3.45	95	1.00	-3.32	100	1.00
5	-3.41	97	1.00	-3.28	102	1.00
6	-3.32	100	1.00	-3.32	100	1.00
7	-3.46	95	1.00	-3.33	100	1.00
8	-3.25	103	1.00	-3.34	99	1.00
Mean	-3.38	98	1.00	-3.30	101	1.00

The mean PCR efficiencies of the GM and target taxon-specific systems were 93% and 106% for MON 89034, 105% and 109% for 1507, 102% and 106% for MON 88017, and 98% and 101% for 59122, respectively. The R^2 of the methods was 0.99 or 1.00 for both systems in all cases. Overall, the data reported confirmed the appropriate PCR efficiency and linearity of the four methods tested on MON 89034 x 1507 x MON 88017 x 59122 maize samples.

7. Method performance requirements

The results of the verification study for the MON 89034, 1507, MON 88017 and 59122 detection methods applied to event MON 89034 x 1507 x MON 88017 x 59122 maize DNA are reported in Tables 6, 7, 8 and 9, respectively. Results were evaluated with respect to the method acceptance criteria, as established by ENGL and adopted by the EURL-GMFF (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>, see also Annex 1). In addition, Tables 6 to 9 report the trueness and repeatability standard deviation for each GM level for all methods.

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 89034 method on event MON 89034 x 1507 x MON 88017 x 59122 maize DNA.

MON 89034					
Unknown sample GM%	Expected value (GMO%)				
	0.09	0.4	0.9	3.0	8.0
Mean	0.09	0.38	0.84	2.69	7.52
SD	0.01	0.05	0.07	0.31	0.90
RSD _r (%)	12	14	8.0	11	12
Bias (%)	-2.2	-4.2	-6.2	-10	-6.1

Table 7. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the 1507 method on event MON 89034 x 1507 x MON 88017 x 59122 maize DNA.

1507					
Unknown sample GM%	Expected value (GMO%)				
	0.1	0.5	0.9	2.0	5.0
Mean	0.09	0.41	0.81	1.64	4.78
SD	0.01	0.06	0.07	0.10	0.47
RSD _r (%)	13	15	8.4	6.3	9.8
Bias (%)	-12	-18	-9.8	-18	-4.4

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 88017 method on event MON 89034 x 1507 x MON 88017 x 59122 maize DNA.

MON 88017					
Unknown sample GM%	Expected value (GMO%)				
	0.09	0.5	0.9	5.0	8.0
Mean	0.07	0.43	0.75	4.66	6.89
SD	0.01	0.05	0.07	0.54	0.57
RSD _r (%)	9.3	11	8.9	12	8.2
Bias (%)	-24	-15	-16	-6.8	-14

Table 9. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the 59122 method on event MON 89034 x 1507 x MON 88017 x 59122 maize DNA.

59122					
Unknown sample GM%	Expected value (GMO%)				
	0.1	0.4	0.9	2.0	4.5
Mean	0.12	0.41	1.02	1.97	4.63
SD	0.02	0.03	0.06	0.09	0.29
RSD_r (%)	15	8.4	5.8	4.7	6.4
Bias (%)	24	1.3	14	-1.3	3.0

The trueness of the method is estimated using the measures of the method bias for each GM level. According to the ENGL acceptance criteria and method performance requirements, the trueness of the method, measured as bias from the accepted value, should be $\pm 25\%$ across the entire dynamic range. As shown in tables 6, 7, 8 and 9, the values ranged from -10% to -2.2% for MON 89034, from -18% to -4.4% for 1507, from -24% to -6.8% for MON 88017, and from -1.3% to 24% for 59122. Therefore, the four methods satisfied the above mentioned requirement throughout their respective dynamic ranges.

Tables 6 to 9 further document the relative repeatability standard deviation (RSD_r) as estimated for each GM level. In order to accept methods for collaborative trial evaluation, the EURL-GMFF requires that RSD_r values are below 25%. As it can be observed from Tables 6 to 9, the values ranged between 8.0% and 14% for MON 89034, between 6.3% and 15% for 1507, between 8.2% and 12% for MON 88017, and between 4.7% and 15% for 59122. Therefore, the four methods satisfied this requirement throughout their respective dynamic ranges.

8. Comparison of method performance between event MON 89034 x 1507 x MON 88017 x 59122 and the single trait events

An indicative comparison of the four methods performances on the maize event MON 89034 x 1507 x MON 88017 x 59122 and the single trait events is shown in Tables 10, 11, 12 and 13. The performance of the methods on the single lines was previously assessed through international collaborative trials.

Table 10. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the MON 89034 detection method on event MON 89034 x 1507 x MON 88017 x 59122 and on event MON 89034.

Trueness and repeatability of MON 89034 quantification on MON 89034 x 1507 x MON 88017 x 59122			Trueness and repeatability of MON 89034 quantification on single event MON 89034*		
GM%	Bias (%)	RSD_r (%)	GM%	Bias (%)	RSD_r (%)
0.09	-2.2	12	0.09	25	18
0.4	-4.2	14	0.4	6.4	13
0.9	-6.2	8.0	0.9	4.3	17
3.0	-10	11	3.0	-5.8	12
8.0	-6.1	12	8.0	-11	9.5

*method validated (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>)

Table 11. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the 1507 detection method on event MON89034 x 1507 x MON88017 x 59122 and on event 1507.

Trueness and repeatability of 1507 quantification on MON 89034 x 1507 x MON 88017 x 59122			Trueness and repeatability of 1507 quantification on single event 1507*		
GM%	Bias (%)	RSD_r (%)	GM%	Bias (%)	RSD_r (%)
0.1	-12	13	0.1	6.0	18
0.5	-18	15	0.5	-4.0	12
0.9	-9.8	8.4	0.9	3.7	7.7
2.0	-18	6.3	2.0	-1.7	8.5
5.0	-4.4	9.8	5.0	8.4	14

*method validated (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>)

Table 12. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the MON 88017 detection method on event MON 89034 x 1507 x MON 88017 x 59122 and on event MON 88017.

Trueness and repeatability of MON 88017 quantification on MON 89034 x 1507 x MON 88017 x 59122			Trueness and repeatability of MON 88017 quantification on single event MON 88017*		
GM%	Bias (%)	RSD_r (%)	GM%	Bias (%)	RSD_r (%)
0.09	-24	9.3	0.09	-2.6	28
0.5	-15	11	0.5	2.9	13
0.9	-16	8.9	0.9	-9.6	19
5.0	-6.8	12	5.0	-4.8	19
8.0	-14	8.2	8.0	-7.6	18

*method validated (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>)

Table 13. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the 59122 detection method on event MON 89034 x 1507 x MON 88017 x 59122 and on event 59122.

Trueness and repeatability of 59122 quantification on MON 89034 x 1507 x MON 88017 x 59122			Trueness and repeatability of 59122 quantification on single event 59122*		
GM%	Bias (%)	RSD_r (%)	GM%	Bias (%)	RSD_r (%)
0.1	24	15	0.1	29	18
0.4	1.3	8.4	0.4	15	14
0.9	14	5.8	0.9	9.0	16
2.0	-1.3	4.7	2.0	7.0	14
4.5	3.0	6.4	4.5	-1.0	8.5

*method validated (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>)

Regarding trueness, the MON 89034 method (Table 10), when applied to event MON 89034 x 1507 x MON 88017 x 59122 and compared to the single line, showed lower bias at 0.09%, 0.4% and 8% GM levels and higher bias at 0.9% and 3% GM levels. The 1507 event-specific method (Table 11) showed generally lower bias when applied to the single event, apart from the highest GM level (5.0%). The MON 88017 even-specific method (table 12) showed lower bias through the entire dynamic range when applied to the single line compared to the stacked event. Finally, the 59122 method (Table 13) when applied to the stack event showed lower bias at the 0.1%, 0.4 and 2.0% GM levels and higher at 0.9% and 4.5%, compared with the single line. In all cases, the trueness of the four event-specific methods when applied to the stacked event was within the acceptance range set by ENGL ($\pm 25\%$) on the whole dynamic ranges studied.

Concerning the relative repeatability standard deviation (RSD_r %), the MON 89034 method (Table 10) showed lower values at 0.09%, 0.9% and 3.0% GM levels when applied to the stacked and compared to the single event, and higher values at 0.4% and 8%. The 1507 method (Table 11), showed lower values at 0.1%, 2.0% and 5.0% and higher to 0.5% and 0.9%, when applied to the stacked event. The MON 88017 and the 59122 methods (Tables 12 and 13) showed better precision over the whole dynamic range when applied to the stacked event compared to the single lines. In all cases, the relative repeatability standard deviation (RSD_r %) of the four event-specific methods, when applied to the stack event, was below the ENGL acceptance level established at maximum 25%.

Therefore, the method verification has demonstrated that the MON 89034, 1507, MON 88017 and 59122 detection methods, developed to detect and quantify the single events, can be equally applied for the quantification of the respective events combined in event MON 89034 x 1507 x MON 88017 x 59122.

9. Conclusions

The overall method performance of the four event-specific methods for the quantitative detection of events MON 89034, 1507, MON 88017 and 59122 combined in maize event MON 89034 x 1507 x MON 88017 x 59122 have been evaluated with respect to the method acceptance criteria and the method performance requirements recommended by the ENGL (as detailed under <http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>), and to the validation results obtained for the single trait events (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>).

The results obtained during the present verification study indicate that the analytical modules of the methods submitted by the applicant comply with ENGL performance criteria. The methods are therefore applicable to the control samples provided (see paragraph 3 "Materials"), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

10. Quality assurance

The EURL-GMFF carries out its operations according to ISO 9001 (certificate number: CH-32231) and ISO 17025 (certificate number: DAC-PL-0459-06-00) [DNA extraction, qualitative and quantitative PCR in the area of Biology (DNA extraction and PCR method validation for the detection and identification of GMOs in food and feed materials)].

11. References

1. Horwitz W., 1995. Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem*, 67: 331-343.
2. International Standard (ISO) 5725:1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization.

12. Annex 1: method acceptance criteria and method performance requirements as set by the European Network of GMO Laboratories (ENGL)

Method Acceptance Criteria should be fulfilled at the moment of submission of a method (Phase 1: acceptance for the collaborative study).

Method Performance Requirements should be fulfilled in a collaborative study in order to consider the method as fit for its purpose (Phase 2: evaluation of the collaborative study results).

Method Acceptance Criteria

Applicability

Definition: The description of analytes, matrices, and concentrations to which a method can be applied.

Acceptance Criterion: The applicability statement should provide information on the scope of the method and include data for the indices listed below for the product/s for which the application is submitted. The description should also include warnings to known interferences by other analytes, or inapplicability to certain matrices and situations.

Practicability

Definition: The ease of operations, the feasibility and efficiency of implementation, the associated unitary costs (e.g. Euro/sample) of the method.

Acceptance Criterion: The practicability statement should provide indication on the required equipment for the application of the method with regards to the analysis *per se* and the sample preparation. An indication of costs, timing, practical difficulties and any other factor that could be of importance for the operators should be indicated.

Specificity

Definition: Property of a method to respond exclusively to the characteristic or analyte of interest.

Acceptance Criterion: The method should be event-specific and be functional only with the GMO or GM based product for which it was developed. This should be demonstrated by empirical results from testing the method with non-target transgenic events and non-transgenic material. This testing should include closely related events and cases where the limit of the detection is tested.

Dynamic Range

Definition: The range of concentrations over which the method performs in a linear manner with an acceptable level of accuracy and precision.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least 5 times the target concentration. Target concentration is intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below. The range of the standard curve(s) should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) end.

Accuracy

Definition: The closeness of agreement between a test result and the accepted reference value.

Acceptance Criterion: The accuracy should be within $\pm 25\%$ of the accepted reference value over the whole dynamic range.

Amplification Efficiency

Definition: The rate of amplification that leads to a theoretical slope of -3.32 with an efficiency of 100% in each cycle. The efficiency of the reaction can be calculated by the following equation: Efficiency = $[10^{(-1/\text{slope})}] - 1$.

Acceptance Criterion: The average value of the slope of the standard curve should be in the range of $(-3.1 \geq \text{slope} \geq -3.6)$.

R² Coefficient

Definition: The R² coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: The average value of R² should be ≥ 0.98 .

Repeatability Standard Deviation (RSD_r)

Definition: The standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time.

Acceptance Criterion: The relative repeatability standard deviation should be below 25% over the whole dynamic range of the method.

Note: Estimates of repeatability submitted by the applicant should be obtained on a sufficient number of test results, at least 15, as indicated in ISO 5725-3 (1994).

Limit of Quantitation (LOQ)

Definition: The limit of quantitation is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy.

Acceptance Criterion: LOQ should be less than 1/10th of the value of the target concentration with an RSD_r $\leq 25\%$. Target concentration should be intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below.

Limit of Detection (LOD)

Definition: The limit of detection is the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified, as demonstrated by single laboratory validation.

Acceptance Criterion: LOD should be less than $1/20^{\text{th}}$ of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring $\leq 5\%$ false negative results. Target concentration should be intended as the threshold relevant for legislative requirements.

Robustness

Definition: The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.

Acceptance Criterion: The response of an assay with respect to these small variations should not deviate more than $\pm 30\%$. Examples of factors that a robustness test could address are: use of different instrument type, operator, brand of reagents, concentration of reagents, and temperature of reaction.

Method Performance Requirements***Dynamic Range***

Definition: In the collaborative trial the dynamic range is the range of concentrations over which the reproducibility and the trueness of the method are evaluated with respect to the requirements specified below.

Acceptance Criterion: The dynamic range of the method should include the $1/10$ and at least five times the target concentration. Target concentration should be intended as the threshold relevant for legislative requirements.

Reproducibility Standard Deviation (RSD_R)

Definition: The standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where test results are obtained with the same method, on identical test items, in different laboratories, with different operators, using different equipment. Reproducibility standard deviation describes the inter-laboratory variation.

Acceptance Criterion: The relative reproducibility standard deviation should be below 35% at the target concentration and over the entire dynamic range. An $RSD_R < 50\%$ is acceptable for concentrations below 0.2%.

Trueness

Definition: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Acceptance Criterion: The trueness should be within $\pm 25\%$ of the accepted reference value over the whole dynamic range.



Event-specific Method for the Quantification of Maize Line MON 89034 Using Real-time PCR

Protocol

21 October 2008

**Joint Research Centre
Institute for Health and Consumer Protection
Biotechnology & GMOs Unit**

Method development:

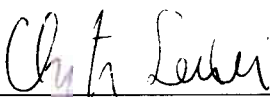
Monsanto Company

Collaborative trial:

Community Reference Laboratory for GM Food and Feed (CRL-GMFF)
Biotechnology & GMOs Unit

Drafted by

C. Savini

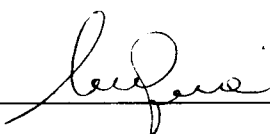


Report Verification Team

1) M. Ermolli



2) M. Querci



Scientific and technical approval

M. Mazzara




Compliance to CRL Quality System

S. Cordeil



Authorisation to publish

G. Van den Eede



Address of contact laboratory:

European Commission, Joint Research Centre

Institute for Health and Consumer Protection (IHCP)

Biotechnology and GMOs Unit – Community Reference Laboratory for GM Food and Feed

Via Fermi 2749, 21027 Ispra (VA) - Italy

Content

1. GENERAL INFORMATION AND SUMMARY OF THE METHOD	4
2. VALIDATION STATUS AND PERFORMANCE CHARACTERISTICS	4
2.1 GENERAL	4
2.2 COLLABORATIVE TRIAL	5
2.3 LIMIT OF DETECTION (LOD)	5
2.4 LIMIT OF QUANTIFICATION (LOQ)	5
2.5 MOLECULAR SPECIFICITY	5
3. PROCEDURE	6
3.1 GENERAL INSTRUCTIONS AND PRECAUTIONS	6
3.2 REAL-TIME PCR FOR QUANTITATIVE ANALYSIS OF MAIZE EVENT MON 89034	7
3.2.1 General	7
3.2.2 Calibration	7
3.2.3 Real-time PCR set-up	7
3.3 DATA ANALYSIS	9
3.4 CALCULATION OF RESULTS	9
4. MATERIALS	10
4.1 EQUIPMENT	10
4.2 REAGENTS	10
4.3 PRIMERS AND PROBES	10

1. General information and summary of the method

This protocol describes an event-specific real-time quantitative TaqMan[®] PCR procedure for the determination of the relative content of maize event MON 89034 DNA to total maize DNA in a sample.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction vessels.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the specific detection of maize event MON 89034 DNA, a 77-bp fragment of the integration region of the construct inserted into the plant genome (located at the 3' insert-to-plant junction) is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye FAM as a reporter at its 5' end and with the non-fluorescent quencher MGBNFQ (minor groove binding non-fluorescent quencher) at its 3' end.

For the relative quantification of maize event MON 89034 DNA, a maize specific reference system amplifies a 79-bp fragment of the maize endogenous *hmg* gene (high mobility group), using a two specific primers and an *hmg* gene-specific probe labelled with FAM and TAMRA as described above.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of event MON 89034 DNA in a test sample, MON 89034 and *hmg* Ct values are determined for the sample. Standard curves are then used to estimate the relative amount of maize event MON 89034 DNA to total maize DNA.

2. Validation status and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from maize leaves, seeds and grains containing mixtures of genetically modified and conventional maize.

The reproducibility and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GMO contents.

2.2 Collaborative trial

The method was validated in an international collaborative study by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with twelve participating laboratories in November 2007.

Each participant received twenty blind samples containing maize MON 89034 genomic DNA at five GM contents, ranging from 0.09% to 8.0%.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM maize MON 89034 in four unknown samples. Four replicates of each GM level were analysed on the same PCR plate.

A detailed validation report can be found at <http://gmo-crl.jrc.it/statusofdoss.htm>

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.04% in 200 ng of total maize DNA. The relative LOD was not assessed in a collaborative study.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.085% in 200 ng of total maize DNA. The lowest relative GM content of the target sequence included in collaborative trial was 0.09 %.

2.5 Molecular specificity

According to the method developer, the method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome. The sequence is specific to maize event MON 89034 and thus imparts event-specificity to the method.

The specificity of event-specific assay was experimentally tested in real-time PCR by the applicant against DNA extracted from plant materials containing the specific targets of Roundup Ready® canola (RT200), Roundup Ready® canola (RT73), conventional canola, Roundup Ready® maize (GA21), Roundup Ready® maize (NK603), YieldGard® corn borer maize (MON 810), YieldGard® Rootworm/Roundup Ready® maize (MON 88017), YieldGard® Rootworm maize (MON 863), lysine maize (LY038), MON 89034 maize, conventional maize, Roundup Ready® cotton (MON 1445), Bollgard® cotton (MON 531), Bollgard® cotton (MON 757), BollgardII® cotton (MON 15985), MON 88913 cotton, conventional cotton, Roundup Ready® soybean 40-3-2, MON 89788 soybean, conventional soybean, Roundup Ready® wheat (MON71800), conventional wheat, lentil, quinoa, sunflower nuts, buckwheat, pinenuts, rye berries, millet, peanut (shelled).

None of the GM-lines tested, except the positive control maize line MON 89034, produced detectable amplification signals.

The specificity of the maize reference assay *hmg* was experimentally tested by the applicant against DNA extracted from plant materials containing Roundup Ready® canola (RT200), Roundup Ready® canola (RT73), conventional canola, Roundup Ready® maize (GA21), Roundup Ready® maize (NK603), YieldGard® corn borer maize (MON 810), YieldGard® Rootworm/Roundup Ready® maize (MON 88017), YieldGard® rootworm maize (MON863), lysine maize (LY038), MON 89034 maize, conventional maize, Roundup Ready® cotton (MON 1445), Bollgard® cotton (MON 531), Bollgard® cotton (MON 757), BollgardII® cotton (MON 15985), MON 88913 cotton, conventional cotton, Roundup Ready® soybean 40-3-2, MON 89788 soybean, conventional soybean, Roundup Ready® wheat (MON71800), conventional wheat, lentil, sunflower, buckwheat, rye berries, peanut.

None of the samples tested, except the control maize lines GA21, NK603, MON 810, MON 863, MON 88017, LY038, MON 89034 and conventional maize, produced detectable amplification signals.

3. Procedure

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Laboratory organisation, e.g. “forward flow direction” during PCR-setup, should follow the guidelines given by relevant authorities, e.g. ISO 24276:2006.
- PCR-reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
- All the equipment used should be sterilised prior to use and any residue of DNA has to be removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.
- Filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed frequently.
- Laboratory benches and equipment should be cleaned periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps - unless specified otherwise - should be carried out at 0-4°C.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of maize event MON 89034

3.2.1 General

The PCR set-up for the taxon specific target sequence (*hmg*) and for the GMO (event MON 89034) target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of maximum 200 ng of template DNA per reaction well is recommended.

The method is developed for a total volume of 50 µL for the MON 89034 reaction and of 25 µL for the *hmg* reaction mixture with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

The calibration curves consist of five samples. The first point of the calibration curves is a 10% MON 89034 maize DNA in non-GM maize DNA for a total of 200 ng of DNA (corresponding to approximately 73394 maize genome copies with one genome assumed to correspond to 2.725 pg of haploid maize genomic DNA) ⁽¹⁾.

A calibration curve is produced by plotting the Ct values against the logarithm of the target copy number for the calibration points. This can be done e.g. by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the sequence detection system software.

The copy number measured for the unknown sample DNA is obtained by interpolation from the standard curves.

3.2.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run.
Keep thawed reagents at 1-4°C on ice.
2. In two reaction tubes (one for the MON 89034 system and one for the *hmg* system) on ice, add the following components (Tables 1 and 2) in the order mentioned below (except DNA) to prepare the master mixes.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the MON 89034 specific system.

Component	Final concentration	µL/reaction
TaqMan® 2x PCR Master Mix	1x	25
MON 89034 primer 1 (10 µM)	450 nM	2.25
MON 89034 primer 2 (10 µM)	450 nM	2.25
MON 89034 probe (5 µM)	100 nM	1.00
Nuclease free water	#	15.5
Template DNA (max 200 ng)	#	4.0
Total reaction volume:		50

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the maize *hmg* reference system.

Component	Final concentration	µL/reaction
Nuclease free water	#	8.95
TaqMan® buffer A (10x)	1x	2.5
MgCl ₂ (25 mM)	6.5 mM	6.5
dNTP mix (10 mM each)	200 µM each	0.5
AmpliTaq Gold polymerase (5 U/µl)	1.25 U	0.25
<i>hmg</i> primer 1 (10 µM)	300 nM	0.75
<i>hmg</i> primer 2 (10 µM)	300 nM	0.75
<i>hmg</i> probe (5 µM)	160 nM	0.8
Template DNA (max 200 ng)	#	4.0
Total reaction volume:		25

- Mix gently and centrifuge briefly.
- Prepare two reaction tubes (one for the MON 89034 event and one for the *hmg* master mixes) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
- Add to each reaction tube the correct amount of master mix (e.g. 46 x 3 = 138 µL master mix for three PCR repetitions for the MON 89034 reactions and 21 x 3 = 63 µL for *hmg* reactions). Add to each tube the correct amount of DNA (e.g. 4 x 3 = 12 µL DNA for three PCR repetitions). Vortex each tubes for approx. 10 sec. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.
- Spin down the tubes in a microcentrifuge. Aliquot 50 µL (or 25 depending on the reaction mixture) in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250 x *g* for 1 minute at 4°C to room temperature) to spin down the reaction mixture.

7. Place the plate into the instrument.
8. Run the PCR with cycling conditions described in Table 3:

Table 3. Cycling program for maize MON 89034 and *hmg* systems

Step	Stage	T°C	Time (sec)	Acquisition	Cycles
1	UNG	50°C	120	No	1
2	Initial denaturation	95°C	600	No	1
3	Amplification	Denaturation	95°C	No	45
		Annealing & Extension	60°C	Yes	

3.3 Data analysis

Subsequent to the real-time PCR, analyse the run following the procedure below:

- a) set the threshold: display the amplification curves of one system (e.g. MON 89034) in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no “fork effect” between repetitions of the same sample. Press the “update” button to ensure changes affect Ct values. Switch to the linear view mode by clicking on the Y axis of the amplification plot, and check that the threshold previously set falls within the geometric phase of the curves.
- b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at $Ct = 25 - 3 = 22$).
- c) Save the settings.
- d) Repeat the procedure described in a) and b) on the amplification plots of the other system (e.g. *hmg* system).
- e) Save the settings and export all the data to a text file for further calculations.

3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument's software calculates the Ct values for each reaction.

The standard curves are generated both for the *hmg* and the MON 89034 specific systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample DNA.

For the determination of the amount of MON 89034 maize DNA in the unknown sample, the copy number of the GMO is divided by the copy number of the maize reference gene (*hmg*) and multiplied by 100 to obtain the percentage value ($GM\% = \text{MON 89034}/hmg \times 100$).

4. Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 mL reaction tubes

4.2 Reagents

- TaqMan® 2X PCR Master Mix, Applied Biosystems Part No 4304437
- TaqMan® 1000X Rxn Gold/Buffer A Pack (10x) Applied Biosystems Part No 4304441

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
MON 89034 target sequence	
MON 89034 primer 1	5' – TTC TCC ATA TTG ACC ATC ATA CTC ATT– 3'
MON 89034 primer 2	5' – CGG TAT CTA TAA TAC CGT GGT TTT TAA A– 3'
MON 89034 (Probe)	6-FAM 5' – ATC CCC GGA AAT TAT GTT – 3' MGBNFQ
Reference gene <i>hmg</i> target sequence	
<i>hmg</i> primer 1	5' – TTG GAC TAG AAA TCT CGT GCT GA– 3'
<i>hmg</i> primer 2	5' – GCT ACA TAG GGA GCC TTG TCC T – 3'
<i>hmg</i> (Probe)	6-FAM 5' – CAA TCC ACA CAA ACG CAC GCG TA – 3' TAMRA



EUROPEAN COMMISSION
DIRECTORATE GENERAL JRC
JOINT RESEARCH CENTRE
INSTITUTE FOR HEALTH AND CONSUMER PROTECTION
COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED



Event-specific method for the quantitation of maize line TC1507 using real-time PCR

Protocol

Method development:




Pioneer Hi-Bred International
GeneScan Analytics GmbH

Method validation:

Joint Research Centre – European Commission
Biotechnology & GMOs Unit

Contents

1. GENERAL INFORMATION AND SUMMARY OF THE METHODOLOGY.....	4
2. VALIDATION STATUS AND PERFORMANCE CHARACTERISTICS.....	4
2.1 GENERAL	4
2.2 COLLABORATIVE TRIAL	5
2.3 LIMIT OF DETECTION.....	5
2.4 LIMIT OF QUANTITATION	5
2.5 MOLECULAR SPECIFICITY	5
3. PROCEDURES.....	6
3.1 GENERAL INSTRUCTIONS AND PRECAUTIONS	6
3.2 REAL-TIME PCR FOR QUANTITATIVE ANALYSIS OF TC1507 MAIZE	7
3.4.1 General	7
3.4.2 Calibration	7
3.4.3 Real-time PCR set-up	7
3.3 DATA ANALYSIS	9
3.4 CALCULATION OF RESULTS	10
4. MATERIALS.....	10
4.1 EQUIPMENT	10
4.2 REAGENTS.....	11
4.3 PRIMERS AND PROBES	11
5. BUFFERS AND SOLUTIONS	12
6. REFERENCES	12

<u>Document Approval</u>		
Name / Function	Date	Signature
Marco Mazzara <i>Sector Head</i>	21/02/2005	
Stephane Cordeil <i>Quality Manager</i>	21/02/2005	
Guy Van den Eede <i>B&GMOs Unit Head</i>	21/02/2005	

Address of contact laboratory:

European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Biotechnology and GMOs Unit – Community Reference Laboratory
Via Fermi 1, 21020 Ispra (VA) - Italy

1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan[®] PCR procedure for the determination of the relative content of event TC1507 DNA to total maize DNA in a sample.

The PCR assay has been optimised for use in real-time PCR instruments for plastic reaction vessels. Glass capillaries are not recommended for the buffer composition described in this method.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to the use in PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run, use of DNA spikes) are recommended.

For specific detection of event TC1507 genomic DNA, a 58-bp fragment of the recombination region of parts of the construct inserted into the plant genome is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5' end and TAMRA as a quencher dye at its 3' end.

For relative quantitation of event TC1507 DNA, a maize-specific reference system amplifies a 79-bp fragment of HMG (High Mobility Group) gene, a maize endogenous gene, using a pair of HMG gene-specific primers and an HMG gene-specific probe labelled with FAM and TAMRA as described above.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantitation of the amount of event TC1507 DNA in a test sample, event TC1507 and HMG Ct values are determined for the sample. Standard curves are then used to calculate the relative content of event TC1507 DNA to total maize DNA.

2. Validation status and performance characteristics

2.1 General

The method has been optimised for ground maize seed, containing mixtures of genetically modified TC1507 and conventional maize.

The reproducibility and trueness of the method was tested through collaborative trial using samples at different GMO contents.

2.2 Collaborative trial

The method was validated in a collaborative trial by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with 14 laboratories.

Each participant received twelve unknown samples. The samples consisted of DNA mixtures of 0% and 100% TC1507 maize genomic DNA at six GMO levels, between 0.0 % and 5.0 %.

Each test sample was analyzed by PCR in three repetitions. The study was designed as a blind duplicate collaborative trial; each laboratory received each level of GM TC1507 in two unknown samples, and the two replicates for each GM level were analyzed on the same PCR plate.

A detailed validation report can be found under <http://gmo-crl.jrc.it/statusofdoss.htm>

2.3 Limit of detection

According the method developer, the absolute LOD of the method is 1.25 copies (8 positives out of 10 replicates). The relative LOD was not assessed in a collaborative trial. The lowest relative concentration of the target sequence included in collaborative trail was 0.1%.

2.4 Limit of quantitation

According the method developer, the relative LOQ of the method is $\leq 0.08\%$. The absolute LOQ for the individual systems is ≤ 10 copies (TC1507) and ≤ 40 copies (HMG). The lowest relative concentration of the target sequence included in collaborative trail was 0.1%.

2.5 Molecular specificity

The method utilizes a unique DNA sequence of the recombination region of parts of the construct inserted into the plant genome. The sequence is specific to TC1507 and thus imparts event-specificity to the detection method.

The specificity was assessed by Blastsearch on 16/11/2002. No 100% match with other maize GMO sequences was found.

The specificity was experimentally tested against DNA extracted from plant materials containing the specific targets of TC1360, Bt176, GA21, NK603, MON810, Bt11, Starlink,

T25, MON 863 maize, Roundup Ready® soybean, conventional rapeseed, rice and wheat. None of the materials yielded detectable amplification.

The target sequence is a single copy sequence in the haploid TC1507 genome.

3. Procedures

3.1 General instructions and precautions

- All handling of reagents and controls should occur in an ISO 9001:2000 or ISO 17025 environment or equivalent.
- The procedures require experience of working under sterile conditions.
- Laboratory organization, e.g. "flow direction" during PCR-setup, should follow the guidelines given by relevant authorities like e.g. ISO, CEN, Codex alimentarius commission.
- PCR-reagents shall be stored and handled in a separate room and freezer and in equipment where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment – especially pipettes.
- All the equipment used must be sterilized prior to use and any residue of DNA has to be removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and shall not adsorb protein or DNA.
- In order to avoid contamination, filter pipette tips protected against aerosol should be used.
- Use only powder-free gloves and change them frequently.
- Clean lab-benches and equipment periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps - unless specified otherwise - shall be carried out at 0 - 4°C.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of TC1507 maize

3.4.1 General

The PCR set-up for the taxon specific target sequence (HMG) and for the GMO (TC1507) target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of maximum 200 ng of template DNA per reaction well is recommended.

The method is developed for a total volume of 25 µl per reaction mixture with the reagents as listed in Table 1 and Table 2.

3.4.2 Calibration

Separate calibration curves with each primer/probe system are generated in the same analytical amplification run.

The calibration curves consist of four dilutions of a DNA sample containing 10% TC1507. A series of one to five dilution intervals at a starting concentration of 73,394 maize genome copies may be used (corresponding to 200 ng of DNA with one maize genome assumed to correlate to 2.725 pg of haploid maize genomic DNA) (Arumuganathan & Earle, 1991).

A calibration curve is produced by plotting Ct-values against the logarithm of the target copy number for the calibration points. This can be done e.g. by use of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the sequence detection system software.

The copy numbers measured for the unknown sample DNA is obtained by interpolation from the standard curves.

3.4.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run. **Keep thawed reagents at 1-4°C on ice.**
2. In two reaction tubes (one for TC1507 system and one for the HMG system) on ice, add the following components (Tables 1 and 2) in the order mentioned below (except DNA) to prepare the master mixes.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the reference HMG specific system.

Component	Final concentration	µl/reaction
Buffer 10x (including Rox)	1x	2.5 µl
Primer MaiJ-F1	300 nM	-
Primer mhmg-rev	300 nM	-
Probe mhmg	180 nM	-
MgCl ₂ 25 mM	4.5 mM	4.5 µl
dNTPs ^a 10/20 mM	200/400 µM	0.5 µl
AmpliTaq Gold Polymerase	1U/reaction	-
Nuclease free water		up to 25 µl
[Template DNA (maximum 200 ng, see 3.4.1 and 3.4.2)]		(5 µl)
Total reaction volume:		25 µl

^a dATP (10 mM), dCTP (10 mM), dGTP (10 mM), dUTP (20 mM)

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for TC1507 specific system.

Component	Final concentration	µl/reaction
Buffer 10x (including Rox)	1x	2.5 µl
Primer MaiY-F1	300 nM	-
Primer MaiY-R3	300 nM	-
Probe MaiY-S1	150 nM	-
MgCl ₂ 25 mM	5.5 mM	5.5 µl
dNTPs ^a 10/20 mM	200/400 µM	0.5 µl
AmpliTaq Gold Polymerase	1U/reaction	
Nuclease free water		up to 25 µl
[Template DNA (maximum 200 ng, see 3.4.1 and 3.4.2)]		(5 µl)
Total reaction volume:		25 µl

^a dATP (10 mM), dCTP (10 mM), dGTP (10 mM), dUTP (20 mM)

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the TC1507 and one for the HMG master mix) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
5. Add to each reaction tube the correct amount of master mix (e.g. $20 \times 3 = 60 \mu\text{l}$ master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. $5 \times 3 = 15 \mu\text{l}$ DNA for three PCR repetitions). Low-speed vortex each tubes at least three times for approx 30 sec. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.
6. Spin down the tubes in a micro-centrifuge. Aliquot 25 μl in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately $250 \times g$ for 1 minute at 4 °C to room temperature) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with cycling conditions described in Table 3:

Table 3. Reaction conditions.

Step	Stage	T °C	Time (sec)	Acquisition	Cycles
1	Initial denaturation	95 °C	600"	No	1x
2a	Denaturation	95 °C	15"	No	45x
2b	Amplification Annealing & Extension	60 °C	60"	Measure	

3.3 Data analysis

Subsequent to the real-time PCR, analyse the run following the procedure below:

- a) Set the threshold: display the amplification curves of one system (e.g. TC1507) in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no "fork effect" between repetitions of the same sample. Press the update button to ensure changes affect Ct values. Switch to the linear view mode by clicking on the Y axis of the amplification plot, and check that the threshold previously set falls within the geometric phase of the curves.

b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at $Ct = 25 - 3 = 22$).

c) Save the settings

d) Repeat the procedure described in a) and b) on the amplification plots of the other system (e.g. HMG system).

e) Save the settings and export all the data into an Excel file for further calculations.

3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instruments software calculated the Ct-values for each reaction.

The standard curves are generated both for the HMG and TC1507 specific systems by plotting the Ct-values measured for the calibration points against the logarithm of the DNA copy numbers, and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample DNA by interpolation from the standard curves.

For the determination of the amount of TC1507 DNA in the unknown sample, the TC1507 copy number is divided by the copy number of the maize reference gene (HMG) and multiplied by 100 to obtain the percentage value ($GM\% = TC1507/HMG * 100$).

4. Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for evaluating data after standard curve method (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge

- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 ml tubes

4.2 Reagents

(equivalents may be substituted)

- TRIS pH=8.0: Tris[hydroxymethyl] aminomethane hydrochloride (Molecular Biology grade)
- KOAc (SIGMA Part No P1190)
- Gelatine (VWR Part No 1.04078.1000)
- Tween 20 (SIGMA Part No P9416-50ML)
- Glycerol (SIGMA Part No P5516-100ML)
- Rox (Applied Biosystems Part No 434925)
- dATP (GeneCraft Part No GC-013-007)
- dCTP (GeneCraft Part No GC-013-009)
- dGTP (GeneCraft Part No GC-013-006)
- dUTP (GeneCraft Part No GC-013-010)
- MgCl₂ (SIGMA Part No M1028-1ML)
- Ampli Taq Gold (Applied Biosystems Part No N8080242)

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
<i>TC1507 target sequence</i>	
MaiY-F1	TAG TCT TCG GCC AGA ATG G
MaiY-R3	CTT TGC CAA GAT CAA GCG
MaiY-S1	6-FAM-TAA CTC AAG GCC CTC ACT CCG-TAMRA
<i>Reference gene HMG target sequence</i>	
MaiJ-F2	TTG GAC TAG AAA TCT CGT GCT GA
mhmg-rev	GCT ACA TAG GGA GCC TTG TCC T
Mhmg-probe	6-FAM-CAA TCC ACA CAA ACG CAC GCG TA-TAMRA

5. Buffers and Solutions

The following describes the preparation of the buffers used in this procedure. Volume may be scaled as needed. Equivalent reagent may be substituted.

- **Preparation of the 10x Buffer**

a) Mix the following chemicals at the final concentration indicated and adjust the buffer to pH = 8.0

Component	Final concentration
Tris pH = 8.0	0.5 M
KOAc	0.5 M
Gelatine	0.5%
Tween 20	0.1%
Glycerol	0.8%
Rox	0.2 µl/reaction
Water	-

6. References

Arumuganathan, K., Earle, E.D. (1991). Nuclear content of some important plant species. Plant Mol Biol Reporter 9, 208-218.



Event-specific Method for the Quantification of Maize Line MON 88017 Using Real-time PCR

Protocol

30 March 2010

**Joint Research Centre
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit**

Method development:

Monsanto Company

Collaborative trial:

Community Reference Laboratory for GM Food and Feed (CRL-GMFF)
Molecular Biology and Genomics Unit

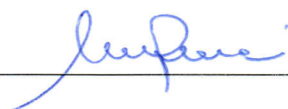
Drafted by

A. Bogni




Report Verification Team

1) M. Querci




2) M. Ermolli




Scientific and technical approval

M. Mazzara



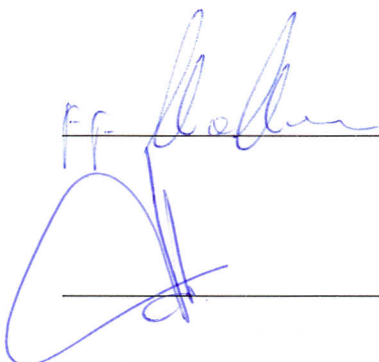
Compliance to CRL Quality System

S. Cordeil



Authorisation to publish

G. Van den Eede



Address of contact laboratory:

European Commission, Joint Research Centre

Institute for Health and Consumer Protection (IHCP)

Molecular Biology and Genomics Unit – Community Reference Laboratory for GM Food and Feed

Via Fermi 2749, 21027 Ispra (VA) - Italy

Content

1. GENERAL INFORMATION AND SUMMARY OF THE METHOD	4
2. VALIDATION STATUS AND PERFORMANCE CHARACTERISTICS	4
2.1 GENERAL	4
2.2 COLLABORATIVE TRIAL	5
2.3 LIMIT OF DETECTION (LOD)	5
2.4 LIMIT OF QUANTIFICATION (LOQ)	5
2.5 MOLECULAR SPECIFICITY	5
3. PROCEDURE	6
3.1 GENERAL INSTRUCTIONS AND PRECAUTIONS	6
3.2 REAL-TIME PCR FOR QUANTITATIVE ANALYSIS OF MON 88017 MAIZE	7
3.2.1 General	7
3.2.2 Calibration	7
3.2.3 Real-time PCR set-up	7
3.3 DATA ANALYSIS	9
3.4 CALCULATION OF RESULTS	10
4. MATERIALS	10
4.1 EQUIPMENT	10
4.2 REAGENTS	10
4.3 PRIMERS AND PROBES	11
5. REFERENCES	11

1. General information and summary of the method

This protocol describes an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event MON 88017 DNA to total maize DNA in a sample.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction vessels.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the specific detection of maize event MON 88017 DNA, a ~~94-bp~~ 95-bp fragment of the integration region of the construct inserted into the plant genome (located at the ~~5'~~ 3' flanking DNA region) is amplified using two specific primers. PCR products are measured at each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye 6-FAM as a reporter at its 5' end and with TAMRA as a quencher dye at its 3' end. The 5' nuclease activity of the Taq DNA polymerase results in the specific cleavage of the probe, leading to increased fluorescence, which is then monitored.

For the relative quantification of maize event MON 88017 DNA, a maize-specific reference system amplifies a 79-bp fragment of the maize endogenous *hmg* gene (*high mobility group*), using two specific primers and a *hmg* gene-specific probe labelled with 6-FAM and TAMRA as described above.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of event MON 88017 DNA in a test sample, MON 88017 and *hmg* Ct values are determined for the sample. Standard curves are then used to estimate the relative amount of maize event MON 88017 DNA to total maize DNA.

2. Validation status and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from maize seeds and grains containing mixtures of genetically modified and conventional maize.

The reproducibility and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GMO contents (%).

2.2 Collaborative trial

The method was validated in an international collaborative study by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with twelve laboratories in July 2007.

Each participant received twenty blind samples containing MON 88017 genomic DNA at five GM contents, ranging from 0.09% to 8.0%.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM MON 88017 in four unknown samples. Four replicates of each GM level were analysed on the same PCR plate.

A detailed validation report can be found at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>

2.3 Limit of detection (LOD)

According to the data provided by the applicant, the relative LOD of the method is at least 0.045% in 200 ng of total maize DNA. The relative LOD was not assessed in collaborative study.

2.4 Limit of quantification (LOQ)

According to the data provided by the applicant, the relative LOQ of the method is at least 0.09% in 200 ng of total maize DNA. The lowest relative GM content of the target sequence included in the international collaborative study was 0.09%.

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome. The sequence is specific to event MON 88017 and thus imparts event-specificity to the method.

The specificity of MON 88017 assay (forward/reverse primers and probe) was experimentally tested in real-time PCR by the applicant against DNA extracted from samples containing Roundup Ready[®] maize MON 88017 (positive control), Roundup Ready[®] canola (RT200), Roundup Ready[®] canola (RT73), conventional canola, Roundup Ready[®] maize (GA21), Roundup Ready[®] maize (NK603), YieldGard[®] Corn Borer maize (MON810), YieldGard[®] Rootworm maize (MON863), Lysine maize (LY038), conventional maize, Roundup Ready[®] cotton (MON 1445), Bollgard[®] cotton (MON 531), Bollgard[®] cotton (MON 757), BollgardII[®] cotton (MON 15985), Roundup Ready[®] Flex cotton (MON 88913), conventional cotton, Roundup Ready[®] soybean (40-3-2), conventional soybean, Roundup Ready[®] wheat (MON 71800), conventional wheat, lentil, sunflower, buckwheat, rye berries and peanut.

According to the applicant, none of the GM lines tested, except the positive control MON 88017, produced amplification signals.

The specificity of the maize reference assay *hmg* was experimentally tested by the applicant against DNA extracted from samples containing Roundup Ready® corn MON 88017, Roundup Ready® canola (RT200), Roundup Ready® canola (RT73), conventional canola, Roundup Ready® maize (GA21), Roundup Ready® maize (NK603), YieldGard® Corn Borer maize (MON810), YieldGard® Rootworm maize (MON863), Lysine maize (LY038), conventional maize, Roundup Ready® cotton (MON 1445), Bollgard® cotton (MON 531), Bollgard® cotton (MON 757), BollgardII® cotton (MON 15985), Roundup Ready® Flex cotton (MON 88913), conventional cotton, Roundup Ready® soybean (40-3-2), conventional soybean, Roundup Ready® wheat (MON 71800), conventional wheat, lentil, sunflower, buckwheat, rye berries and peanut.

Only the positive control maize line MON 88017 and Roundup Ready® maize (GA21), Roundup Ready® maize (NK603), YieldGard® Corn Borer maize (MON810), YieldGard® Rootworm maize (MON863), Lysine maize (LY038) and conventional maize produced amplification signals.

3. Procedure

3.1 General instructions and precautions

- The procedures require sterile conditions working experience.
- Laboratory organisation, e.g. “forward flow direction” during PCR-setup, should follow international guidelines, e.g. ISO 24276:2006.
- PCR-reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
- Equipment used should be sterilised prior to use and any residue of DNA should be removed. All material used (e.g. vials, containers, pipette tips, etc.) should be suitable for PCR and molecular biology applications; it should be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.
- Filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed frequently.
- Laboratory benches and equipment should be cleaned periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.

- All handling steps - unless specified otherwise - should be carried out at 0–4°C.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of MON 88017 maize

3.2.1 General

The PCR set-up for the taxon specific target sequence (*hmg*) and for the GMO (event MON 88017) target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of maximum 200 ng of template DNA per reaction well is recommended.

The method is developed, for MON 88017, in a total volume of 50 µL per reaction mixture with the reagents as listed in Table 1 and, for *hmg*, in a total volume of 25 µL per reaction mixture with the reagents as listed in and Table 2.

3.2.2 Calibration

The calibration curves consist of five samples. The first point of the calibration curves is a 10% MON 88017 in non-GM maize DNA for a total of 200 ng of DNA (corresponding to approximately 73394 maize genome copies with one genome assumed to correspond to 2.725 pg of haploid maize genomic DNA) ⁽¹⁾.

A calibration curve is produced by plotting the Ct values against the logarithm of the target copy number for the calibration points. This can be done e.g. by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the sequence detection system software.

The copy number measured for the unknown sample DNA is obtained by interpolation from the standard curves.

3.2.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run.
Keep thawed reagents at 1-4°C on ice.
2. In two reaction tubes (one for the MON 88017 system and one for the *hmg* system) on ice, add the following components (Tables 1 and 2) in the order mentioned below (except DNA) to prepare the master mixes.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the MON 88017 specific system.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
MON 88017 AF (10 µM)	150 nM	0.75
MON 88017 AR (10 µM)	150 nM	0.75
MON 88017 AP (5 µM)	50 nM	0.50
Nuclease free water	#	19
Template DNA (max 200 ng)	#	4.0
Total reaction volume:		50

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the maize *hmg* reference system.

Component	Final concentration	µL/reaction
Buffer A (10x)	1x	2.5
<i>Hmg</i> F (10 µM)	300 nM	0.75
<i>hmg</i> R (10 µM)	300 nM	0.75
<i>hmg</i> P (5 µM)	160 nM	0.80
MgCl ₂ (25 mM)	6.5 mM	6.5
dNTPs mix (10 mM)	200 µM	0.5
Ampli TAQ Gold	1.25 U/rnx	0.25
Nuclease free water	#	8.95
Template DNA (max 200 ng)	#	4.0
Total reaction volume:		25

- Mix gently and centrifuge briefly.
- Prepare two reaction tubes (one for the MON 88017 and one for the *hmg* master mixes) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
- Add to each reaction tube the correct amount of master mix (e.g. 46 x 3 = 138 µL master mix for three PCR repetitions for MON 88017 and 21 x 3 = 63 µL master mix for three PCR repetitions for *hmg*). Add to each tube the correct amount of DNA (e.g. 4 x 3 = 12 µL DNA for three PCR repetitions). Vortex each tubes for approx. 10 sec. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.

6. Spin down the tubes in a microcentrifuge. Aliquot 50 µL in each well for MON 88017 and 25 µL for *hmg*. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250 x *g* for 1 minute at 4 °C to room temperature) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with cycling conditions described in Table 3:

Table 3. Cycling program for MON 88017 and maize *hmg* system

Step	Stage	T °C	Time (sec)	Acquisition	Cycles
1	UNG	50 °C	120	No	1
2	Initial denaturation	95 °C	600	No	1
3	Amplification	Denaturation 95 °C	15	No	45
		Annealing & Extension 60 °C	60	Yes	

3.3 Data analysis

Subsequent to the real-time PCR, analyse the run following the procedure below:

- a) Set the threshold: display the amplification curves of one system (e.g. MON 88017) in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no “fork effect” between repetitions of the same sample. Press the “update” button to ensure changes affect Ct values. Switch to the linear view mode by clicking on the Y axis of the amplification plot, and check that the threshold previously set falls within the geometric phase of the curves.
- b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at Ct = 25 – 3 = 22).
- c) Save the settings.
- d) Repeat the procedure described in a) and b) on the amplification plots of the other system (e.g. *hmg* system).
- e) Save the settings and export all the data to a text file for further calculations.

3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument's software calculates the Ct values for each reaction.

The standard curves are generated both for the *hmg* and the MON 88017 specific systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample DNA.

For the determination of the amount of event MON 88017 DNA in the unknown sample, the MON 88017 copy number is divided by the copy number of the maize reference gene (*hmg*) and multiplied by 100 to obtain the percentage value ($GM\% = \text{MON 88017}/hmg \times 100$).

4. Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Analysis software
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 mL reaction tubes

4.2 Reagents

- TaqMan[®] Universal PCR Master Mix (2X). Applied Biosystems Part No. 4304437
- 0.5 M EDTA. Sigma Cat. No. E-7647-01-0
- PCR Nucleotide Mix (10mM dNTPs). Promega Cat. No. C114G
- TaqMan[®] 1000X Rxn Gold/Buffer A Pack. Applied Biosystems Cat. No. 4304441
- AmpliTaq Gold Polymerase. Applied Biosystems Cat. No. N808-0244
- Nuclease-free water. Sigma Cat. No. W-4502
- 1 M Tris-HCl, pH 8.0. Sigma Cat. No. T-3038

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
MON 88017 target sequence	
MON 88017 AF	5' – GAG CAG GAC CTG CAG AAG CT – 3'
MON 88017 AR	5' – TCC GGA GTT GAC CAT CCA – 3'
MON 88017 AP (Probe)	6-FAM-TCC CGC CTT CAG TTT AAA CAG AGT CGG GT-TAMRA
Reference gene hmg target sequence	
<i>Hmg</i> F	5' – TTG GAC TAG AAA TCT CGT GCT GA– 3'
<i>Hmg</i> R	5' – GCT ACA TAG GGA GCC TTG TCC T– 3'
<i>hmg</i> P (Probe)	6-FAM- CAA TCC ACA CAA ACG CAC GCG TA -TAMRA

5. References

1. Arumuganathan K., Earle E. D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9: 208-218.



EUROPEAN COMMISSION
DIRECTORATE GENERAL JRC
JOINT RESEARCH CENTRE
INSTITUTE FOR HEALTH AND CONSUMER PROTECTION
COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED



Event-specific method for the quantitation of maize 59122 using real-time PCR

Protocol

Method development:

Pioneer Hi-Bred International
GeneScan Analytics GmbH

Method validation:

Joint Research Centre – European Commission
Biotechnology & GMOs Unit
Community Reference Laboratory for GM Food and Feed

Contents

1. GENERAL INFORMATION AND SUMMARY OF THE METHODOLOGY	4
2. VALIDATION STATUS AND PERFORMANCE CHARACTERISTICS.....	4
2.1 GENERAL	4
2.2 COLLABORATIVE TRIAL	5
2.3 LIMIT OF DETECTION.....	5
2.4 LIMIT OF QUANTITATION	5
2.5 MOLECULAR SPECIFICITY	5
3. PROCEDURES.....	6
3.1 GENERAL INSTRUCTIONS AND PRECAUTIONS	6
3.2 REAL-TIME PCR FOR QUANTITATIVE ANALYSIS OF DAS-59122-7 MAIZE	7
3.2.1 General	7
3.2.2 Calibration	7
3.2.3 Real-time PCR set-up	7
3.3 DATA ANALYSIS	10
3.4 CALCULATION OF RESULTS	11
4. MATERIALS.....	11
4.1 EQUIPMENT	11
4.2 REAGENTS.....	11
(EQUIVALENTS MAY BE SUBSTITUTED)	11
4.3 PRIMERS AND PROBES	12
5. REFERENCES	12

<u>Document Approval</u>		
Name / Function	Date	Signature
Marco Mazzara <i>Sector Head</i>	08/06/2007	Signed
Stephane Cordeil <i>Quality Manager</i>	08/06/2007	Signed
Guy Van den Eede <i>B&GMOs Unit Head</i>	08/06/2007	Signed

Address of contact laboratory:

European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Biotechnology and GMOs Unit – Community Reference Laboratory
Via Fermi 1, 21020 Ispra (VA) - Italy

1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event DAS-59122-7 DNA to total maize DNA in a sample.

The PCR assay has been optimised for use in real-time PCR instruments for plastic reaction vessels. Glass capillaries are not recommended for the buffer composition described in this method.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to the use in PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run, use of DNA spikes) are recommended.

For specific detection of event DAS-59122-7 genomic DNA, an 86-bp fragment of the recombination region of parts of the construct inserted into the plant genome is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5' end and TAMRA as a quencher dye at its 3' end.

For relative quantitation of event DAS-59122-7 DNA, a maize-specific reference system amplifies a 79-bp fragment of the High Mobility Group (Hmg) gene, a maize endogenous gene, using a pair of Hmg gene-specific primers and an Hmg gene-specific probe labelled with FAM and TAMRA as described above.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantitation of the amount of event DAS-59122-7 DNA in a test sample, event DAS-59122-7 and Hmg Ct values are determined for the sample. Standard curves are then used to calculate the relative content of event DAS-59122-7 DNA to total maize DNA.

2. Validation status and performance characteristics

2.1 General

The method has been optimised for DNA extracted from seeds, containing mixtures of genetically modified and conventional maize.

The reproducibility and trueness of the method was tested through collaborative trial using samples at different GMO contents.

2.2 Collaborative trial

The method was validated in a collaborative trial by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with 14 laboratories.

Each participant received twenty unknown samples containing DAS-59122-7 maize genomic DNA at five concentration levels, between 0.10 % and 4.5 %.

Each test sample was analyzed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM DAS-59122-7 in four unknown samples. Two replicates of each GM level were analyzed on the same PCR plate.

A detailed validation report can be found under <http://gmo-crl.jrc.it/statusofdoss.htm>

2.3 Limit of detection

According to the method developer, the relative LOD of the method is 0.045%. The relative LOD was not assessed in a collaborative trial. The lowest relative concentration of the target sequence included in collaborative trial was 0.10%.

2.4 Limit of quantitation

According to the method developer, the relative LOQ of the method is 0.09%. The lowest relative concentration of the target sequence included in collaborative trial was 0.10%.

2.5 Molecular specificity

The method utilizes a unique DNA sequence of the recombination region of parts of the construct inserted into the plant genome. The sequence is specific to DAS-59122-7 and thus imparts event-specificity to the detection method.

The specificity was assessed by Blastsearch at the National Center for Biotechnology Information (NCBI) with the "Standard nucleotide-nucleotide BLAST [blastn]" (www.ncbi.nlm.nih.gov/blast/Blast.cgi) on the amplicon resulting from the event-specific amplification of the transition region of the sugar beet genomic DNA into the specific event.

No 100% match with other plant GMO sequences was found.

The specificity was experimentally tested against DNA extracted from plant materials containing the specific targets (at least 1000 genomic copies/reaction) of the T25 maize, 1507 maize, Bt176 maize, Bt11 maize, NK603 maize, GA21 maize, MON810 maize, RR

soy, RR rape, potato “new leaf”. In addition DNA extracted from non GM-wheat and -rice was also tested.

None of the materials yielded detectable amplification, apart from the event DAS-59122-7.

3. Procedures

3.1 General instructions and precautions

- All handling of reagents and controls should occur in an ISO 9001:2000 or ISO 17025 environment or equivalent.
- The procedures require experience of working under sterile conditions.
- Laboratory organization, e.g. “flow direction” during PCR-setup, should follow the guidelines given by relevant authorities like e.g. ISO, CEN, Codex alimentarius commission.
- PCR-reagents shall be stored and handled in a separate room and freezer and in equipment where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment – especially pipettes.
- All the equipment used must be sterilized prior to use and any residue of DNA has to be removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and shall not adsorb protein or DNA.
- In order to avoid contamination, filter pipette tips protected against aerosol should be used.
- Use only powder-free gloves and change them frequently.
- Clean lab-benches and equipment periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps - unless specified otherwise - shall be carried out at 0 - 4°C.
- In order to avoid repeated freeze/thaw cycles, aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of DAS-59122-7 maize

3.2.1 General

The PCR set-up for the taxon specific target sequence (Hmg) and for the GMO (DAS-59122-7) target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of maximum of 200 ng of template DNA per reaction well is recommended

The method is developed for a total volume of 25 µl per reaction mixture with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

Separate calibration curves with each primer/probe system are generated in the same analytical amplification run.

The calibration curves consist of four samples. The first point of the calibration curves is a 5% DAS-59122-7 in non-GM maize DNA for a total of 200 ng of DNA (corresponding to 73.394 maize genome copies with one genome assumed to correlate to 2.725 pg of haploid maize genomic DNA) (Arumuganathan & Earle, 1991).

A series of 1:5 dilutions down to 1.6 ng of total maize DNA/sample (S4) starting from S1 may be used.

A calibration curve is produced by plotting Ct-values against the logarithm of the target copy number for the calibration points. This can be done e.g. by use of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the sequence detection system software.

The copy numbers measured for the unknown sample DNA is obtained by interpolation from the standard curves.

3.2.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run. **Keep thawed reagents at 1-4°C on ice.**
2. In two reaction tubes (one for DAS-59122-7 system and one for the Hmg system) on ice, add the following components (Tables 1 and 2) in the order mentioned below (except DNA) to prepare the master mixes.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the reference Hmg specific system.

Component	Final concentration	µl/reaction
PCR buffer II 10x	1x	2.5
Rox Reference Dye (50x)	0.7x	0.35
Tween-20 1%	0.01%	0.25
Glycerol 20%	0.8%	1
dATPs (10 mM)	200 µM	0.5
dCTPs (10 mM)	200 µM	0.5
dGTPs (10 mM)	200 µM	0.5
dUTPs (20 mM)	400 µM	0.5
MgCl ₂ (100 mM)	5.5 mM	1.375
MaiJ-F2 primer (10 µM)	400 nM	1
mhmh-rev primer (10 µM)	400 nM	1
mhmh-probe (10 µM)	150 nM	0.375
Ampli Taq Gold (5U/µl)	0.04 U/µl	0.2
Nuclease free water	#	9.95
Template DNA (see 3.2.1 and 3.2.2)		(5)
Total reaction volume:		25

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for DAS-59122-7 specific system.

Component	Final concentration	µl/reaction
PCR buffer II 10x	1x	2.5
Rox Reference Dye (50x)	0.7x	0.35
Tween-20 1%	0.01%	0.25
Glycerol 20%	0.8%	1
dATPs (10 mM)	200 µM	0.5
dCTPs (10 mM)	200 µM	0.5
dGTPs (10 mM)	200 µM	0.5
dUTPs (20 mM)	400 µM	0.5
MgCl ₂ (100 mM)	5.0 mM	1.25
DAS-59122-7-rb1f primer (10 µM)	250 nM	0.625
DAS-59122-7-rb1r primer (10 µM)	250 nM	0.625
DAS-59122-7-rb1s probe (10 µM)	200 nM	0.5
Ampli Taq Gold (5U/µl)	0.04 U/µl	0.2
Nuclease free water	#	10.7
Template DNA (see 3.2.1 and 3.2.2)		(5)
Total reaction volume:		25

- Mix gently and centrifuge briefly.
- Prepare two reaction tubes (one for the DAS-59122-7 and one for the Hmg master mix) for each DNA sample to be tested (reference curve samples, unknown samples and control samples).
- Add to each reaction tube the correct amount of master mix (e.g. 20 x 3 = 60 µl master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 5 x 3 = 15 µl DNA for three PCR repetitions). Low-speed vortex each tubes at least three times for approx 30 sec. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.
- Spin down the tubes in a micro-centrifuge. Aliquot 25 µl in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g.

approximately 250 x *g* for 1 minute at 4 °C to room temperature) to spin down the reaction mixture.

7. Place the plate into the instrument.
8. Run the PCR with cycling conditions described in Table 3:

Table 3. Reaction conditions.

Step	Stage	T °C	Time (sec)	Acquisition	Cycles
1	Initial denaturation	95 °C	600"	No	1x
2a	Denaturation	95 °C	15"	No	50x
2b	Amplification Annealing & Extension	60 °C	60"	Measure	

3.3 Data analysis

Subsequent to the real-time PCR, analyse the run following the procedure below:

a) Set the threshold: display the amplification curves of one system (e.g. DAS-59122-7) in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no "fork effect" between repetitions of the same sample. Press the update button to ensure changes affect Ct values. Switch to the linear view mode by clicking on the Y axis of the amplification plot, and check that the threshold previously set falls within the geometric phase of the curves.

b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at Ct = 25 – 3 = 22).

c) Save the settings

d) Repeat the procedure described in a) and b) on the amplification plots of the other system (e.g. Hmg system).

e) Save the settings and export all the data into an Excel file for further calculations.

3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instruments software calculated the Ct-values for each reaction.

The standard curves are generated both for the Hmg and DAS-59122-7 specific system by plotting the Ct-values measured for the calibration points against the logarithm of the DNA copy numbers, and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample DNA by interpolation from the standard curves.

For the determination of the amount of DAS-59122-7 DNA in the unknown sample, the DAS-59122-7 copy number is divided by the copy number of the maize reference gene (Hmg) and multiplied by 100 to obtain the percentage value ($GM\% = \text{DAS-59122-7/Hmg} * 100$).

4. Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for evaluating data after standard curve method (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 ml tubes

4.2 Reagents

(equivalents may be substituted)

- PCR buffer II 10x (Applied Biosystems Part No. N808-0019)
- $MgCl_2$ for molecular biology (SIGMA, Part No. M1028-1 ML)

- Rox (Invitrogen Part No 12223-012)
- Tween20 for molecular biology (SIGMA Part No P9416-50 ML)
- Glycerol for molecular biology (minimum 99%) (SIGMA Part No G5516-100 ML)
- dATP (Amersham-Pharmacia Part No 27-2050-02)
- dCTP (Amersham-Pharmacia Part No 27-2060-02)
- dGTP (Amersham-Pharmacia Part No 27-2070-02)
- dUTP (Amersham-Pharmacia Part No 27-2040-01)
- AmpliTaq Gold polymerase (Applied Biosystems Part No N8080240)
- TE-Buffer pH=8.0 (10/1 mM) (Applichem Part No A2575,1000)
- Primers and probes (Metabion)

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
<i>DAS-59122-7 target sequence</i>	
DAS-59122-7-rb1f	5'- GGG ATA AGC AAG TAA AAG CGC TC -3'
DAS-59122-7-rb1r	5'- CCT TAA TTC TCC GCT CAT GAT CAG -3'
DAS-59122-7-rb1s Probe	6-FAM- TTT AAA CTG AAG GCG GGA AAC GAC AA-TAMRA-3'
<i>Reference gene Hmg target sequence</i>	
MaiJ-F2	5'- TTG GAC TAG AAA TCT CGT GCT GA -3'
mhmg-Rev	5'- GCT ACA TAG GGA GCC TTG TCC T -3'
Mhmg probe	6-FAM- CAA TCC ACA CAA ACG CAC GCG TA -TAMRA

5. References

Arumuganathan, K., Earle, E.D. (1991). Nuclear content of some important plant species. Plant Mol Biol Reporter 9, 208-218.

European Commission

EUR 24706 EN– Joint Research Centre – Institute for Health and Consumer Protection

Title: Report on the Verification of the Performance of MON 89034, 1507, MON 88017 and 59122 Event-specific Methods on the Maize Event MON 89034 x 1507 x MON 88017 x 59122 Using Real-Time PCR. Validation Report and Protocols

Author(s): E. Luque-Perez, A. Bevilacqua, S. Larcher, G. Pinski, M. Mazzara and G. Van den Eede

Luxembourg: Publications Office of the European Union

2010 – 67 pp. – 21 x 29.7 cm

EUR – Scientific and Technical Research series – ISSN 1018-5593

ISBN 978-92-79-19082-7

doi:10.2788/31795

Abstract

The European Union Reference Laboratory for GM Food and Feed (EURL-GMFF), established by Regulation (EC) No 1829/2003, has carried out a verification study to assess the performance of four quantitative event-specific methods on the maize event MON 89034 x 1507 x MON 88017 x 59122 (unique identifier MON-89Ø34-3 x DAS-Ø15Ø7-1 x MON-88Ø17-3 x DAS-59122-7) which combines the MON 89034, 1507, MON 88017 and 59122 transformation events. The four methods have been validated individually on single-trait events, to detect and quantify each event in maize samples. This study was conducted according to internationally accepted guidelines (1, 2).

In accordance to Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and to Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Monsanto and Dow AgroSciences provided the detection methods and the control samples: genomic DNA from homogenised seeds of MON 89034 x 1507 x MON 88017 x 59122 maize (258-4CC, 258-4JJ, 258-4B, 258-4N) and from homogenised seeds of conventional maize (10001262-V). The EURL-GMFF prepared the verification samples (calibration samples and blind samples at different GM percentages).

The results of the verification study were evaluated with reference to ENGL method performance requirements (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>) and to the validation results on the individual parental events (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>).

The results of this EURL-GMFF verification study are publicly available at <http://gmo-crl.jrc.ec.europa.eu/>.

How to obtain EU publications

Our priced publications are available from EU Bookshop (<http://bookshop.europa.eu>), where you can place an order with the sales agent of your choice.

The Publications Office has a worldwide network of sales agents. You can obtain their contact details by sending a fax to (352) 29 29-42758.

The mission of the JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Union. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.

